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In vitro activity of C-20 methyltransferase, BchU, involved in bacteriochlorophyll *c* biosynthetic pathway in green sulfur bacteria

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Abstract **The activity of a methyltransferase, BchU, which catalyzes methylation at the C-20 position of chlorin ring in the biosynthetic pathway of bacteriochlorophyll *c*, was investigated in vitro. The *bchU* gene derived from the photosynthetic green sulfur bacterium, *Chlorobium tepidum*, was overexpressed in *Escherichia coli* as a His-tagged protein (His_6-BchU), and the enzyme was purified. In the presence of *S*-adenosylmethionine, His_6-BchU methylated zinc bacteriopheophorbide *d* at the C-20 position to give zinc bacteriopheophorbide *c*. Metal-free bacteriopheophorbide *d* could not be methylated by the BchU, indicating that the central metal in the chlorin should be required for the recognition by the BchU.**

Keywords: Bacteriochlorophyll; BchU; Green sulfur bacteria; Methyltransferase; *S*-Adenosylmethionine

1. Introduction

Chlorophyllous pigments serve essential roles for energy-converging systems in photosynthetic organisms. The pigments are assembled into light-harvesting apparatuses and reaction center complexes where sunlight energy is effectively captured and converged to initiate a charge separation process. Photosynthetic green sulfur bacteria have extramembraneous light-harvesting antenna systems, chlorosomes. The constituent pigments of chlorosomes are specialized chlorophyllous pigments, bacteriochlorophyll(BChl)s *c*, *d*, and *e*, which self-aggregated without participation of any proteinous components [1–3]. Another unique characteristic is that there are various homologs in chlorosomal BChls, which differ from each other in the degree of methylation at the 8^2- and 12^1-positions (see R^8/R^{12} in Fig. 1). Such mixed homologs would control the physical sizes of chlorosomes and their optical properties in response to light environments [4].

Another methylation process at the C-20 position, i.e., the conversion of BChl *d* (R^{20} = H) to *c* (R^{20} = CH_3) (see Fig. 1) has been reported to be dependent on growth conditions. Broch-Due and Ormerod [5] observed a light-dependent change in their relative contents and isolated a strain producing mainly BChl *c* from *Chlorobium* (*Chl.*) *vibrioforme* NCIB 8327, which contained BChl *d* as a exclusively pigment in chlorosomes. Recently, we have also isolated a strain producing BChl *c* from the liquid culture of *Chl. vibrioforme* NCIB 8327 containing mainly BChl *d* under low-light conditions [6], and have supposed that the BChl *c* strain was derived from the BChl *d* strain in *Chl. vibrioforme* species by their genetic analyses [7].

The whole genome analysis of *Chl. tepidum* has allowed a draft of the biosynthetic pathway of BChl *c* to be drawn up [8], which has been modified by genetic studies (Fig. 1) [4,9]. A gene encoding C-20 methyltransferase *bchU* was identified by Bryant and his co-workers [10], and they further indicated that the above BChl *d* strains would be produced by a frame-shift inactivation of the *bchU*. Although mutant analyses are useful for the elucidation of in vivo biosynthetic pathways of various (B)Chls in general, the in vitro enzymatic assays are indispensable for conclusive evidence of them to be obtained.

In this study, the *bchU* gene was cloned into the pET-15b vector and overexpressed in *Escherichia coli* BL21(DE3) as a His-tagged protein (His_6-BchU). We purified the His_6-BchU and measured its in vitro enzymatic activity for methylation of some artificial pigments. We will discuss substrate specificity of the BchU along with its role in the biosynthetic pathway of BChl *c*.

2. Materials and methods

2.1. Cloning of bchU gene into expression vector

The *bchU* gene was amplified from the whole genome of *Chl. tepidum* strain WT2321 by PCR using primer set (forward: 5′-TCATATGATGAGCAACAATGACCTCCT-3′ and reverse: 5′-AGGATCCTTACGGCTTCACAGCCTGAA-3′; underlines show *Nde*I and *Bam*HI restriction sites, respectively) and ligated with the *Sma*I-digested pUC118 vector (Takara, Japan) to create the pUC-bchU. Following the digestion of the pUC-bchU with *Nde*I and *Bam*HI, the DNA fragment containing the *bchU* gene was ligated with the pET-15b vector (Novagen, USA) and finally the pET15b-His_6-bchU was obtained.

2.2. Purification of His_6-BchU and SDS–PAGE

The pET15b-His_6-bchU was transformed into *E. coli* BL21(DE3). The His_6-BchU was overexpressed basically according to the method described in a manufactural manual. About 10 g of harvested cells were suspended in buffer A (50 mM Tris/HCl, pH 7.8, 300 mM NaCl,

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Abbreviations: BChl, bacteriochlorophyll; BChlid, bacteriochlorophyllide; BPheid, bacteriopheophorbide; *Chl.*, *Chlorobium*; DLS, dynamic light scattering; HPLC, high performance liquid chromatography; Proto IX, protoporphyrin IX; SAM, *S*-adenosylmethionine

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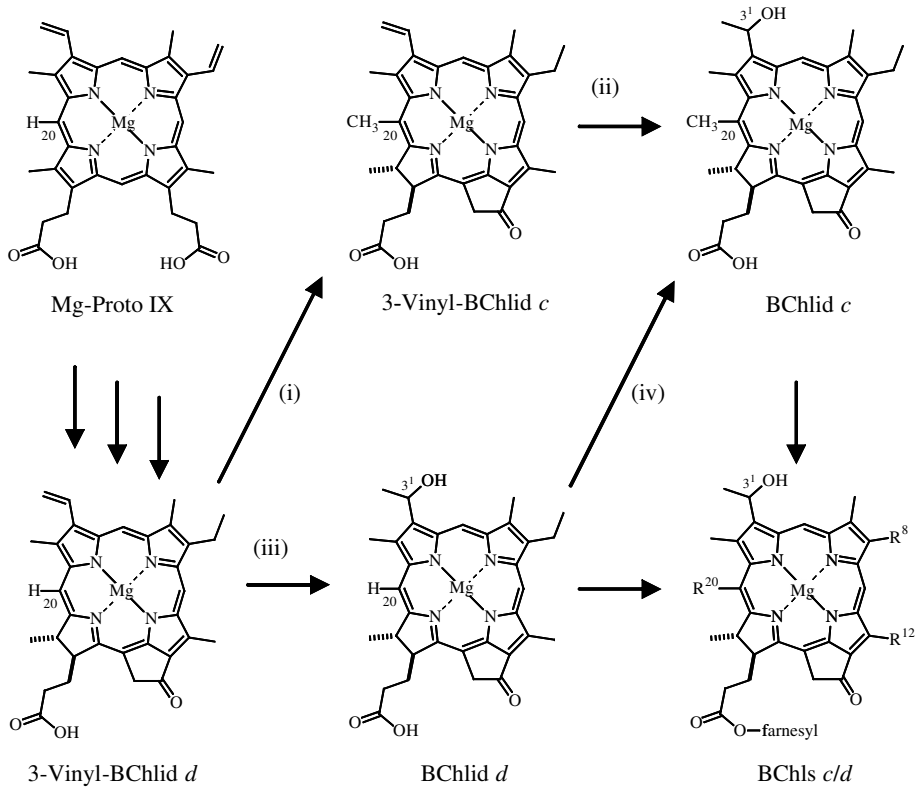


Fig. 1. The schematic diagram of possible biosynthetic pathways of BChls *c* ($R^{20} = CH_3$) and *d* ($R^{20} = H$); the 3^1-stereochemistry is *R* or *S*; $R^8 = C_2H_5$, *n*-C_3H_7, *iso*-C_4H_9; $R^{12} = CH_3$, C_2H_5. Steps (i) and (iv) represent methylation at the C20-position by the BchU, and steps (ii) and (iii) represent hydration of the 3-vinyl group. Additional methylation at the 8^2- and 12^1-positions (R^8 and R^{12} of BChls *c*/*d*) are omitted for simplification.

and 20 mM imidazole). After disruption with sonication followed by centrifugation, the supernatant was mixed with slurry of Ni–NTA agarose (Qiagen, Germany) to adsorb His_6-BchU. After stirring the mixture for 30 min at 4 °C, the agarose was washed batch-wisely with buffer A and then with buffer B (50 mM Tris/HCl, pH 7.8, 300 mM NaCl, and 50 mM imidazole). The adsorbed His_6-BchU proteins were recovered by the elution with buffer C (50 mM Tris/HCl, pH 7.8, 300 mM NaCl, and 250 mM imidazole), and further purified by a gel filtration chromatography using a HiPrep 16/60 Sephacryl S-200 HR column equipped with a ÄKTAexplorer (Amersham Biosciences, USA), which was developed with buffer D (50 mM Tris/HCl, pH 7.8, and 150 mM NaCl) at a flow rate of 0.4 ml/min at 4 °C.

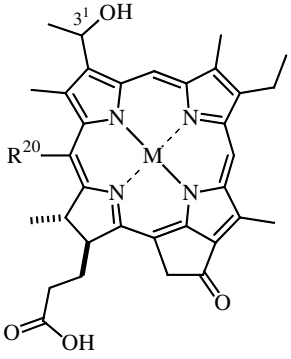
SDS–PAGE was performed according to Laemmli [11]. After electrophoresis, the separated protein bands were stained with Coomassie brilliant blue.

2.3. Preparation of substrates

Chlorophyll *a* was extracted from the cyanobacterium, *Spirulina geitleri*, and converted to methyl 13^2-(demethoxycarbonyl)pheophorbide *a* (methyl pyropheophorbide *a*) in three steps, as described in [12]. Hydration of the 3-vinyl group and hydrolysis of the methyl ester by treatment with 30% hydrogen bromide in acetic acid and then water afforded bacteriopheophorbide (BPheid) *d* (Fig. 2) [13]. BChl *c* which was extracted from a green filamentous bacterium, *Chloroflexus aurantiacus*, was demetallated [13] and hydrolyzed by the action of aqueous HCl [14], to give BPheid *c* (Fig. 2). Metal-free BPheids *d* and *c* were metallated in an acetone solution of $Zn(CH_3COO)_2 \cdot 2H_2O$ [13] to give their zinc complexes, Zn-BPheids *d* and *c*, respectively (Fig. 2). Synthetic pigments were prepared as a mixture of (3^1R) and (3^1S) stereoisomers. Zinc protoporphyrin IX (Zn-Proto IX) was purchased from Wako (Japan).

2.4. BchU assays

A mixture containing His_6-BchU (0.2 μM), artificial pigments (2 μM) and *S*-adenosylmethionine (SAM) (10 μM) in buffer D was incubated at 40 °C for 60 min in the dark. An equal volume of diethyl ether was added and the diethyl ether layer was separated and evaporated. The 17-propionate group of the resulting (Zn-)BPheids was converted to methyl ester by treatment with CH_2N_2/Et_2O. Pigments were analyzed by reverse-phase high performance liquid chromatography (HPLC) (5C18-AR-II, 6 mm∅ × 250 mm, Nacalai Tesque, Japan) with methanol/water (8/2, v/v) at a flow rate of 1.5 ml/min for (Zn-)BPheids or with methanol/acetonitrile/1 M ammonium acetate (425/75/100, v/v/v) at a flow rate of 1.0 ml/min for Zn-Proto IX. HPLC was carried out with the same apparatuses reported previously [6,15].



Zn-BPheid *c*: M = Zn, $R^{20} = CH_3$
Zn-BPheid *d*: M = Zn, $R^{20} = H$
BPheid *c*: M = H_2, $R^{20} = CH_3$
BPheid *d*: M = H_2, $R^{20} = H$

Fig. 2. Molecular structures of (Zn-)BPheids *c* and *d*.

2.5. Other apparatus

Dynamic light scattering (DLS) measurements to estimate molecular-mass sizes of proteins were performed with a DynaPro-MS/MSRC (Proteinsolutions, USA).

3. Results and discussion

3.1. Purification of overexpressed His_6-BchU

Fig. 3 shows the proteins produced in *E. coli* BL21(DE3) cells containing either pET-15b or pET15b-His_6-bchU and purification steps of the His_6-BchU. A protein with an apparent molecular mass of approximately 40 kDa was induced in *E. coli* cells containing pET15b-His_6-bchU as indicated by its binding to Ni–NTA agarose (Fig. 3, lanes 4–6). This molecular mass agrees well with that predicted from the deduced amino acid sequence of the BchU containing a His-tag (MW; 40229.2). A relatively weakly stained protein band showing almost the same mobility as His_6-BchU was detected in a supernatant fraction of control cells containing only pET-15b on SDS–PAGE analysis (Fig. 3, lane 3), but was undoubtedly different from His_6-BchU because it was never recovered from a Ni–NTA agarose.

After disruption of cells followed by centrifugation, His_6-BchU in a supernatant fraction was adsorbed to Ni–NTA agarose and eluted in more than 90% purity as shown in lane 6 of Fig. 3. His_6-BchU was finally purified with a gel filtration chromatography, giving two peaks corresponding to protein sizes of approximately 70 and 123 kDa, respectively, whose molecular masses were estimated in comparison with a calibration curve drawn using molecular-weight markers (data not shown). The SDS–PAGE analysis apparently showed that these two peaks were the identical 40 kDa protein (Fig. 3, lanes 7 and 8). These two peaks were finally considered to be dimer

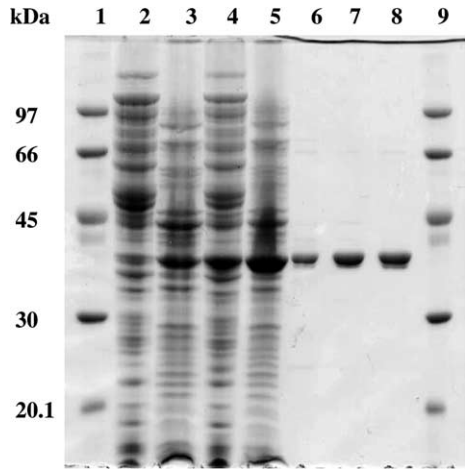


Fig. 3. SDS–PAGE analyses demonstrating the IPTG-induced expression of His_6-BchU in *E. coli* and its purification steps. Lanes 1 and 9, molecular-weight markers; lanes 2 and 3, the precipitate and supernatant fractions recovered after disruption of *E. coli* cells containing pET-15b, respectively; lanes 4 and 5, the same as in lanes 2 and 3 except for *E. coli* cells containing pET15b-His_6-bchU; lane 6, a fraction containing His_6-BchU recovered after Ni–NTA affinity chromatography; lanes 7 and 8, a dimer and tetramer of His_6-BchU purified after gel filtration chromatography, respectively.

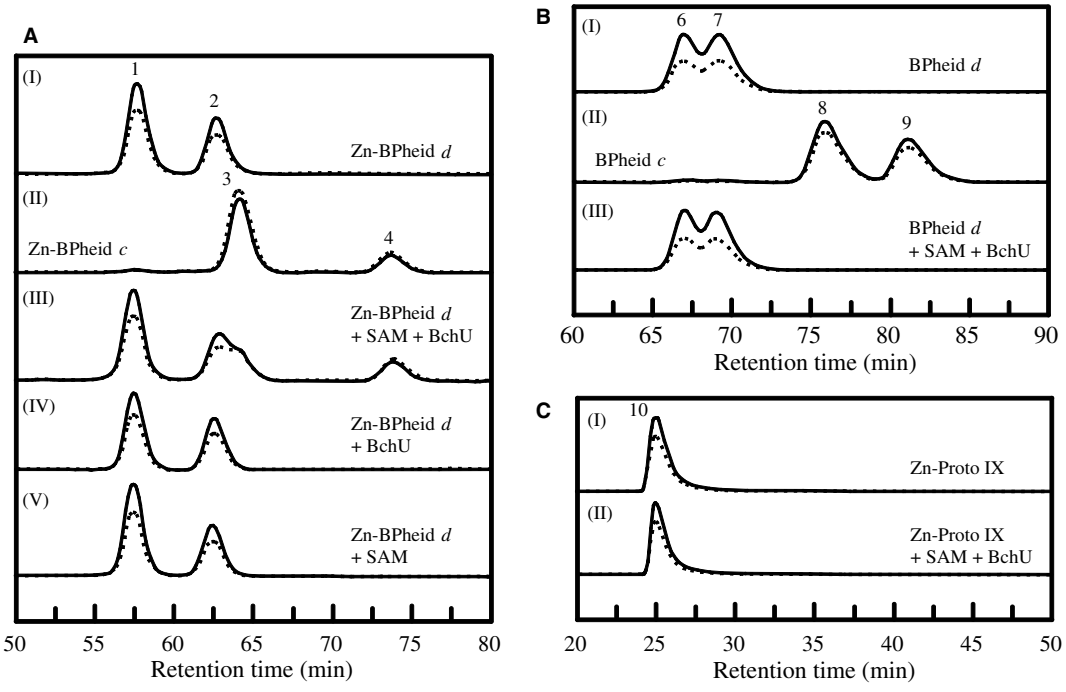


Fig. 4. HPLC analyses of in vitro reactions using a dimeric His_6-BchU in combination with a substrate and/or SAM. All (Zn-)BPheids were esterified at the 17-propionate ($COOH \rightarrow COOCH_3$) before HPLC analyses. Solid/dashed lines depict chromatograms monitored at 424/433, 412/420 and 413/420 nm in (A), (B) and (C), respectively. (A) Zn-BPheid *d* was used as a substrate. (I) and (II) show profiles of methyl esters of Zn-BPheids *d* and c, respectively. (III) is the result after reaction in the presence of BchU, Zn-BPheid *d* and SAM. (IV) and (V) are the results of control experiments without SAM and BchU, respectively. Peaks 1 to 4 represent methyl esters of Zn-(3^1R)- and (3^1S)-BPheids *d*, and Zn-(3^1R)- and (3^1S)-BPheids *c*, respectively. (B) BPheid *d* was used as a substrate. (I) and (II) show profiles of methyl esters of BPheids *d* and *c*, respectively. (III) is the result after reaction in the presence of BchU, BPheids *d* and SAM. Peaks 6–9 represent methyl esters of (3^1R)- and (3^1S) BPheids *d*, and (3^1R)- and (3^1S) BPheids *c*. (C) Zn-Proto IX was used as a substrate. (I) shows a profile of Zn-Proto IX. (II) is the result after reaction in the presence of BchU, Zn-Proto IX and SAM. Peak 10 represents Zn-Proto IX.

and tetramer conformations of His_6-BchU, judged from their DLS measurements (101 and 172 kDa, respectively).

3.2. Methyltransferase activity of BchU in vitro

The enzymatic activity of His_6-BchU was measured in vitro using some artificial pigments. An aqueous reaction mixture containing His_6-BchU (note that we used here a dimer), SAM and a pigment was incubated at 40 °C for 1 h in the dark and the resulting pigments extracted from the mixture were analyzed by HPLC. Since a mixture of (3^1R) and (3^1S) stereoisomers of (Zn-)BPheids *d* was difficult to separate under our HPLC conditions, the mixture was analyzed after esterification at the 17-propionate ($COOH \rightarrow COOCH_3$) to resolve peaks of the two stereoisimers. It is noteworthy that 3^1-epimeric mixtures of (Zn-)BPheids *d* were used as substrates due to the difficulty in their preparative separation.

Fig. 4A shows profiles by HPLC analyses for the experiments using Zn-BPheid *d* as a substrate. The (3^1R) and (3^1S) stereoisomers of Zn-BPheid *d* (peaks 1 and 2 in their methyl esters) were partially converted to new species, which had the same retention times as Zn-BPheid *c* methyl esters (peaks 3 and 4) by His_6-BchU in the presence of SAM (I → III in Fig. 4A). These products were consistent with Zn-BPheid *c* methyl esters from their absorption spectra (λ_{max} = 433 and 664 nm in an HPLC eluent). In the absence of either SAM (Figs. 4A-IV) or His_6-BchU (Figs. 4A-V), Zn-BPheid *c* species could not be detected. These results indicated that the BchU methylated C-20 of both (3^1R) and (3^1S) forms of Zn-BPheid *d* using SAM as a methyl donor. It should be noted that BchU was capable of methylating such an artificial chlorin pigment possessing zinc as a central metal instead of natural magnesium.

In metal-free BPheid *d*, no methyl group was introduced at the C-20 position (Fig. 4B), indicating that a central metal was necessary for the substrate of BchU. The central metal would serve an important role for the substrate to make a proper configuration within an active site of the BchU. Moreover, Zn-Proto IX could not act as a substrate for the BchU as shown in Fig. 4C. The exo-five membered ring would be critically recognized by the BchU.

A tetramer of His_6-BchU exhibited a lower methyltransferase activity compared to that of the dimer (data not shown). Since incubation of the tetramer in a reaction mixture at 40 °C for 1 h induced a partial formation of the dimer (data also not shown), the lower activity in the reaction of the tetramer may be due to the presence of this dimer. Therefore, it is unclear, at present, whether the tetramer has enzymatic activity by itself.

3.3. BchU in biosynthesis pathway of BChl c

From a whole genome analysis of *Chl. tepidum*, Eisen et al. [8] speculated that Mg-Prot IX (see Fig. 1) would be a substrate to be methylated at the C-20 position in the biosynthetic pathway of BChl *c*. Recently, Bryant and his coworkers [4,9] have modified its biosynthetic pathway based on pigment analyses of mutants generated by the inactivation of relevant genes; C-20 methylation of 3-vinyl bacteriochlorophyllide(BChlid) *d* by BchU (see step (i) in Fig. 1) [10]. In the present results, neither Zn-Proto IX nor metal-free pigments were shown to be methylated by the BchU in the presence of SAM, indicating that such a C-20 methylation should occur at the later stage of the BChl *c* biosynthetic pathway after Mg-Proto IX.

The in vitro methylation of Zn-BPheid *d* with 3-(1-hydroxyethyl) group suggested that BChlid *d* would be methylated in vivo at the C-20 position by BchU (step (iv) in Fig. 1). In the BChl *c* biosynthetic pathway, the hydration of the 3-vinyl group would be followed by the 20-methylation (steps (iii) and (iv) in Fig. 1) as an additional bypass for proposed steps (i) and (ii). Moreover, the BchU methylated both (3^1R)- and (3^1S)-epimers of Zn-BPheid *d*, indicating that the BchU could not discriminate the stereochemistry at the 3-(1-hydroxyethyl) group. Therefore, BchU would have broader substrate specificity and catalyze any BChlid *d* homologs other than BChlid *d* possessing 8^2-ethyl and 12^1-methyl groups, used in this study.

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